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Hibernation-Based Therapy to Improve Survival of Severe Blood Loss

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14. ABSTRACT The purpose of these experiments was to find the most effective concentration of melatonin/DMSO that could be administered in conjunction with 4M BHB. Three concentrations were tested, 43mM Melatonin/20% DMSO, 4.3mM Melatonin/2% DMSO and 0.43mM Melatonin/2% DMSO. It was found that 43mM Melatonin/20% DMSO given in conjunction with 4M BHB was the most effective concentration. This concentration, 43mM Melatonin/20% DMSO, when compared to 4.3mM Melatonin/2% DMSO, 0.43mM Melatonin/2% DMSO or lactated Ringers' was associated with a survival benefit. Current studies are focused on finding the Maximum Tolerated Dose (MTD) of 4M BHB/ 43mM Melatonin.					
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Introduction:

Blast injuries have been responsible for the majority of combat deaths in Iraq and Afghanistan, and the likelihood of being exposed to explosives is increasing for military personnel and civilians alike in war zones and other regions of political conflict. The injuries sustained are often accompanied by severe blood loss, and shock from this blood loss is the most common cause of potentially salvageable deaths from combat related injuries.

D-beta hydroxybutyrate and melatonin (BHB/M) is a novel therapy designed to prolong survival in patients who are risk for bleeding to death. Our overall strategy in this series of studies is to capitalize on the physiologic adaptive responses seen in hibernating mammals to aid in salvage of a patient with a potentially life-threatening blood loss, permitting survival to reach effective medical care. BHB/ M includes both an alternate fuel source for cells (D-beta hydroxybutyrate) and a powerful anti-oxidant, melatonin, to protect cells against damage.

Our goal in the current study is to evaluate the safety and efficacy of BHB/M in preclinical animal models leading to human trials. We utilize an animal model of injury simulating battlefield casualty. Our previous work has shown increased survival for both rats and pigs treated with BHB/M. We wish to prove that BHB/M is a safe and effective therapy that can decrease mortality and improve outcomes for injured casualties suffering from polytrauma and blast injuries.

In grant years one and two, Tasks 1-3 were completed. At the completion of Task 1, we determined that 4M BHB/43mM Melatonin was able to be infused using a peripheral vein if the solution was at pH 7.4. Task 2 was done to determine whether 4M BHB/43mM Melatonin infused both intravenously (I.V.) and interosseously (I.O.) was safe. There were no differences between the groups in terms of histopathology, no detrimental effects from intraosseous infusion and no safety issues identified (biochemical, physiologic) out to 14 days after injury /drug administration. Therefore, it was concluded that 4M BHB/43mM Melatonin infused both I.V. and I.O. was safe. Task 3 was done to understand the pharmacokinetics of dosing in anesthetized, non-injured animals. It was observed that there were dose-dependent changes in both serum sodium and potassium. However, no change in pH was observed. It was concluded that I.O.

administration of 4M BHB/43mM Melatonin at 0.66cc/kg/hr resulted in lower circulating levels of BHB and melatonin.

The results presented in this report discuss the results of Task 4a. This task was done to optimize the concentration of melatonin given.

Body:

Task 4a: Observation of the preservation of survival benefit using doses of melatonin at $1/10^{\text{th}}$ and $1/100^{\text{th}}$ the concentration previously studied (43mM melatonin) in conjunction with 4M BHB. Four study groups (Lactated Ringers' (1 Male, 1 Female), 4M BHB/43mM Melatonin (3 Male, 3 Female), 4M BHB/4.3mM Melatonin (3 Male, 3 Female), 4M BHB/0.43mM Melatonin (3 Male, 3 Female)), n=20. All animals will be sacrificed at 72 hours. The following randomization grid was utilized (Table 1).

Table 1.

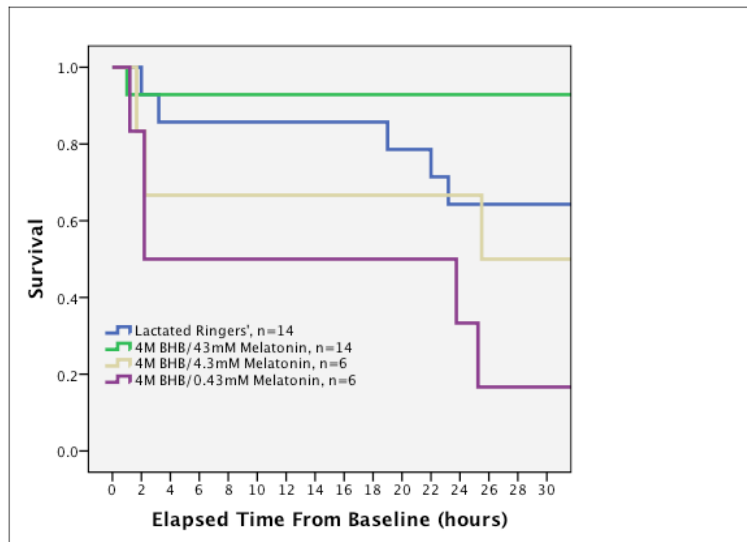
Drug Component	Concentration of Drug component	Animal Sex
Lactated Ringers'	10 cc/kg, 0.66 cc/kg/hr	Male
BHB/M I.V.	4 M BHB/43 mM melatonin	Female
BHB/M I.V.	4 M BHB/4.3 mM melatonin	Male
BHB/M I.V.	4 M BHB/0.43 mM melatonin	Female
BHB/M I.V.	4 M BHB/4.3 mM melatonin	Female
BHB/M I.V.	4 M BHB/0.43 mM melatonin	Male
BHB/M I.V.	4 M BHB/4.3 mM melatonin	Male
BHB/M I.V.	4 M BHB/0.43 mM melatonin	Female
Lactated Ringers'	10 cc/kg, 0.66 cc/kg/hr	Female
BHB/M I.V.	4 M BHB/0.43 mM melatonin	Male
BHB/M I.V.	4 M BHB/0.43 mM	Female

	melatonin	
BHB/M I.V.	4 M BHB/4.3 mM melatonin	Male
BHB/M I.V.	4 M BHB/4.3 mM melatonin	Female
BHB/M I.V.	4 M BHB/43 mM melatonin	Male
BHB/M I.V.	4 M BHB/0.43 mM melatonin	Male
BHB/M I.V.	4 M BHB/4.3 mM melatonin	Female

Experiments were started on 03/04/13 and completed on 08/05/13. In the control groups above (lactated Ringers' (LR), n=2 and 4M d-Betahydroxybutyrate (BHB)/43mM melatonin, n=2) animals resuscitated with lactated Ringers' or 4M BHB/43mM melatonin in a previous phase of the grant were added to the analysis, n=12 and n=12, respectively. Fourteen total animals per group were used for analysis.

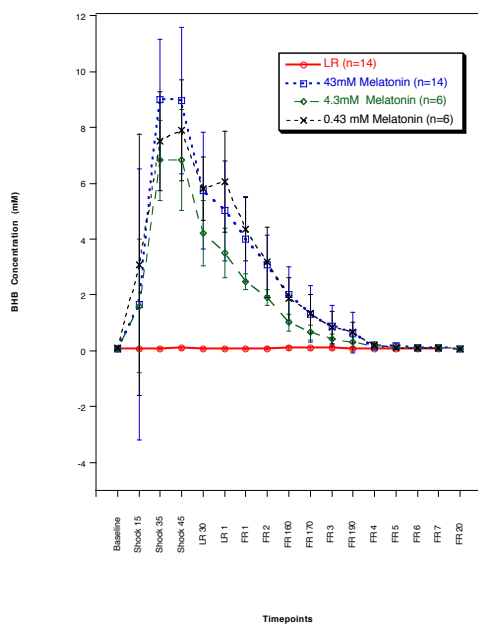
There was no statistical difference in animal weight, volume hemorrhaged or resuscitation fluids given. Survival analysis through the first 30 hours of the experiment show a survival benefit to those animals receiving 4M BHB/43mM melatonin when all groups are compared, $p=0.001$ (Figure 1).

Figure 1. Kaplan-Meier Survival Curve, $p=0.001$ when all groups are compared.



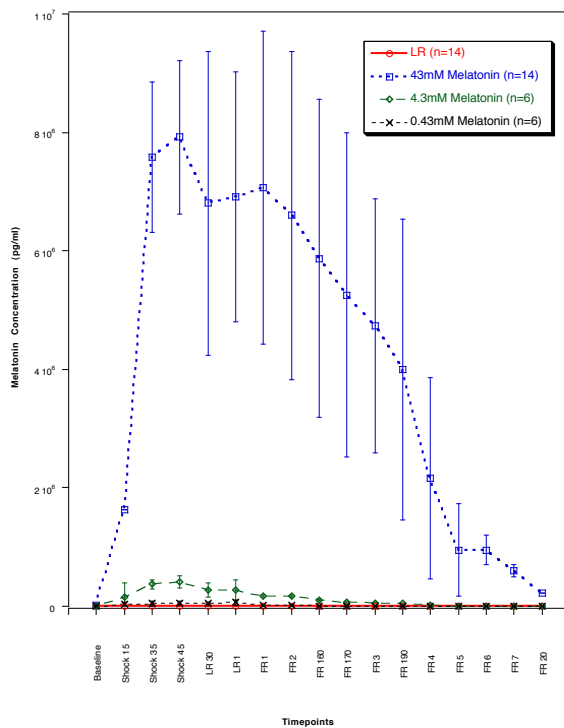
Although all animals received 4M BHB in this phase of the study, BHB serum levels were slightly different, varying as the concentration of the melatonin administered changed (Figure 2).

Figure 2. BHB concentrations of animals undergoing injury, hemorrhagic shock and resuscitation.



Melatonin concentrations significantly changed and melatonin was cleared more rapidly in animals that received either 4.3mM or 0.43mM melatonin (Figure 3).

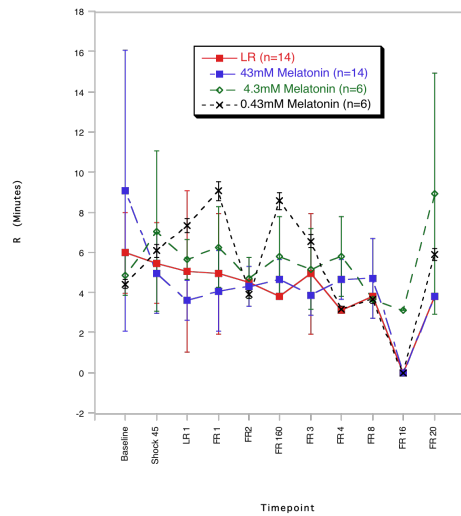
Figure 3. Melatonin concentrations of animals undergoing injury, hemorrhagic shock and resuscitation.



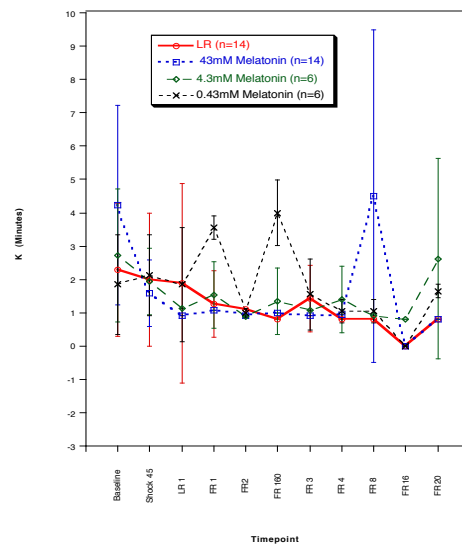
Thromboelastograph (TEG) allows real-time, rapid evaluation of the coagulation status of patients. In our set of experiments, all parameters (Reaction Time (R), K, Angle, and Maximum Amplitude (MA)) exhibited signs of a hypercoagulable state at FR8 that was resolved by FR20 (Figure 4A, B, C, D). This observation is likely associated with resuscitation fluid administration in response to injury and shock (1).

Figure 4. Thrombelastography (TEG) parameters from animals that have received L.R. or 4M BHB and various doses of melatonin, A) R is the time it takes to detect the first levels of clot formation, B) K is the time it takes from the detection of the first levels of clot formation until a fixed level of clot firmness is reached, C) Angle measures the rate of polymerization and D) MA is the measurement of maximum strength or stiffness of the developed clot.

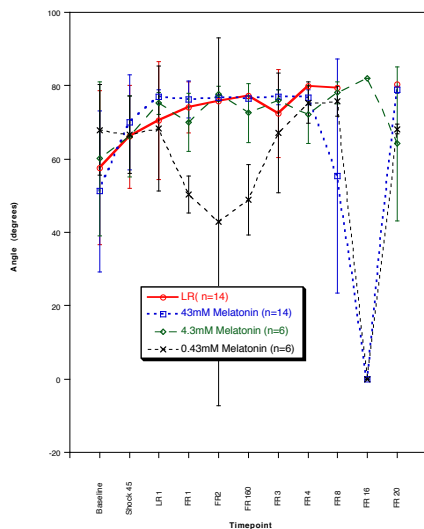
A)



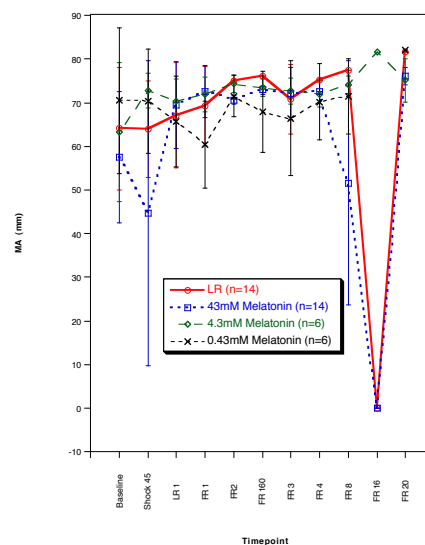
B)



C)



D)



We observed an increase in sodium concentrations with all doses of Melatonin (43mM, 4.3mM, or 0.43mM) when compared to animals receiving L.R. Sodium concentrations returned to the same levels observed in L.R. animals by the FR 20 (Figure 5). Potassium concentrations were increased as a response to shock in all animals while resuscitation brought about a decrease in potassium concentrations in animals receiving all doses of Melatonin (43mM, 4.3mM, or 0.43mM) when compared to animals receiving L.R. (Figure 6). Potassium concentrations were not statistically significant between the groups starting at FR 8 and remained that way until FR 20.

Figure 5. Sodium Concentrations

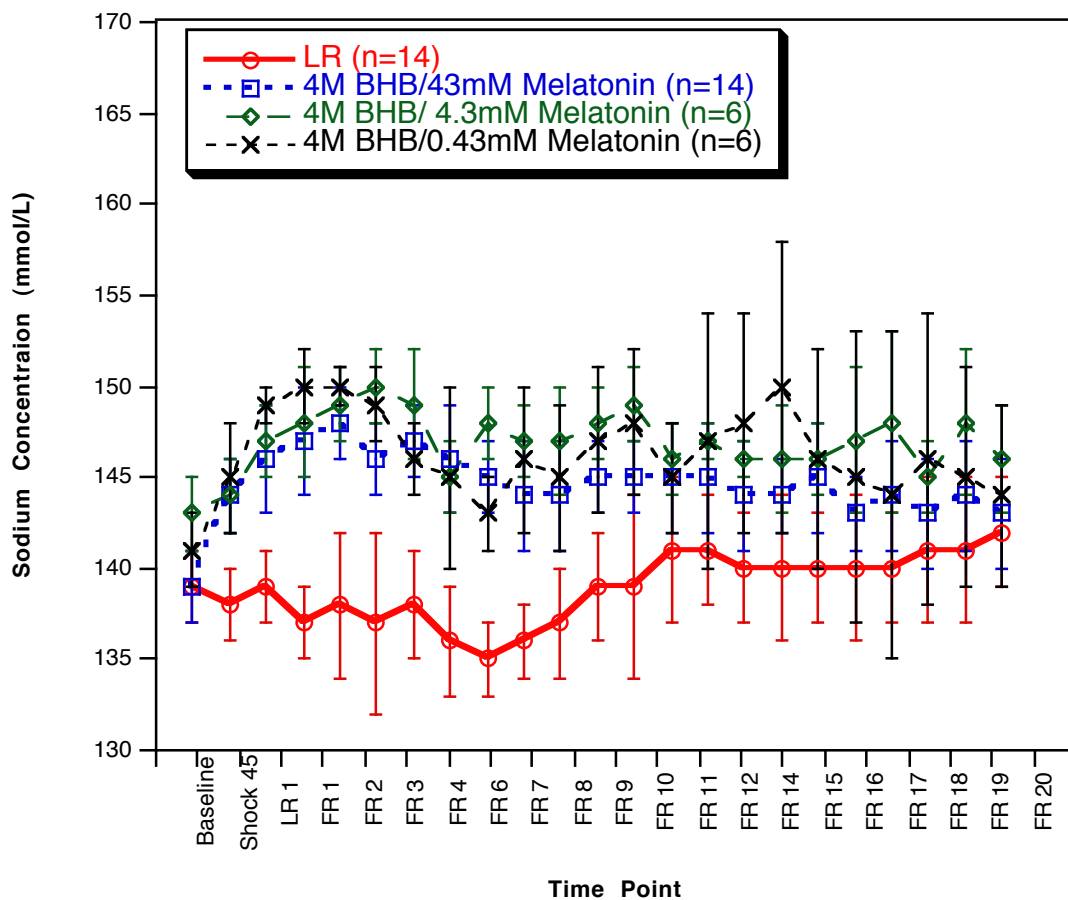
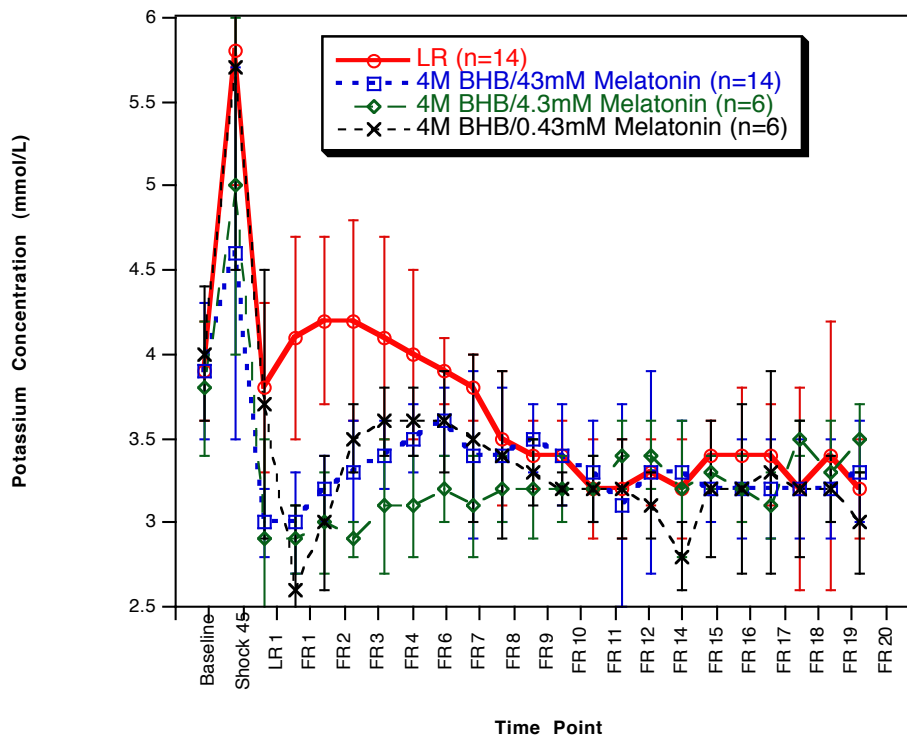


Figure 6. Potassium Concentrations.



At the end of the experiments, necropsies were performed. The following tissue/organs were grossly inspected; adrenal glands, bones, brain, esophagus, eyes gall bladder, heart and great vessels, intestine both large and small, both left and right kidneys, liver, lymph nodes, left and right lung, oral cavity and tongue, pancreas, reproductive tract, skin, sternum bone marrow, stomach, thymus, thyroid, trachea and bronchi, urinary bladder and vessels. Histology was read from the following; heart, rib, tibia (the sight of intraosseous infusion of BHB/M), right and left kidney, right and left lateral liver lobe, thyroid gland, small intestine, large intestine, stomach gall bladder, adrenal glands, thymus pancreas, mesenteric lymph node, submandibular lymph node, urinary bladder, right and left lung, carotid artery, pituitary gland, cervical spinal cord, thoracic spinal cord, lumbar spinal cord, and brain. All necropsies and histology were performed/read either by George Ruth DVM, PhD, DACVP or Nick Robinson, BVSc, PhD, MACVSc, DACVP, Veterinary Pathologists contracted by Experimental Surgical Services.

There were no differences between groups in terms of histopathology.

Task 4b: What is the Maximum Tolerated Dose (MTD) of 4M BHB/43mM Melatonin in our model of shock/polytrauma? Two study groups (I.V. 4M BHB/43mM Melatonin, 2X (3 Male, 3 Female) or I.V. 4M BHB/43mM Melatonin, 4X (3 Male, 3 Female)), n=12 are to be randomized utilizing the below experimental grid (Table 2). Experiments on task 4b began August 2013.

Table 2.

Drug Components	Concentration of Drug	Animal Sex
I.V. BHB/M 2X	4M BHB/43mM Melatonin	Male
I.V. BHB/M 4X	4M BHB/43mM Melatonin	Female
I.V. BHB/M 4X	4M BHB/43mM Melatonin	Female
I.V. BHB/M 2X	4M BHB/43mM Melatonin	Male
I.V. BHB/M 2X	4M BHB/43mM Melatonin	Female
I.V. BHB/M 4X	4M BHB/43mM Melatonin	Female
I.V. BHB/M 2X	4M BHB/43mM Melatonin	Female
I.V. BHB/M 2X	4M BHB/43mM Melatonin	Female
I.V. BHB/M 4X	4M BHB/43mM Melatonin	Male
I.V. BHB/M 4X	4M BHB/43mM Melatonin	Male
I.V. BHB/M 2X	4M BHB/43mM Melatonin	Male
I.V. BHB/M 4X	4M BHB/43mM Melatonin	Male

Key Research Accomplishments:

- * Confirmed survival benefit of treatment with 4M BHB/43mM Melatonin in 20% DMSO in model of shock simulating battlefield injury.
- * In this model, lower doses of melatonin were not associated with survival benefit
- * Currently determining the MTD of 4M BHB/43mM Melatonin in 20% DMSO.

Reportable Outcomes:

- * A manuscript entitled “BHB-M is Safe to Administer Peripherally “ has been submitted to the Journal of Surgical Research for peer review and is in the editing process.
- * A manuscript including data from our I.V. and I.O. administration and pharmacology work is currently being prepared and will be submitted for peer review by the end of the year.

Conclusions:

Melatonin given at 43mM, 4.3mM or 0.43mM in conjunction with 4M BHB was used to treat animals undergoing shock, poly-trauma and resuscitation. Injured animals receiving 4M BHB/43mM melatonin demonstrated a survival benefit when compared to animals receiving 4M BHB/4.3mM Melatonin, 4M BHB/0.43mM Melatonin or L.R. ($p=0.001$, Figure 1.)

We are in the process of determining the MTD of 4M BHB/43mM Melatonin (Table 2).

References:

1. Mulier KE, Greenberg JG, Beilman GJ (2012) Hypercoagulability in porcine hemorrhagic shock is present early after trauma and resuscitation. J Surg Research 174:e31-e35.

Appendices:

Time points defined, Limited Resuscitation (LR)=maintenance of SBP above 80 mmHg, Full Resuscitation (FR)=maintenance of SBP above 90 mmHg, Hgb above 6 and Urine output > 1 cc/kg/hr.

Time point	Elapsed time from Baseline
Baseline	0
Shock 15	15 minutes
Shock 35	35 minutes
Shock 45	45 minutes
LR 30	30 minutes from the start of Limited Resuscitation phase, ~1.5 hours from baseline
LR 1	60 minutes from the start of Limited Resuscitation phase, ~2 hours from baseline
FR 1	1 hour from the start of Full Resuscitation, 2 hours from the start of Limited Resuscitation, ~3 hours from Baseline
FR 2	2 hour from the start of Full Resuscitation, 3 hours from the start of Limited Resuscitation, ~4 hours from Baseline
FR 160	160 minutes from the start of Full Resuscitation, 3 hours 40 minutes from the start of Limited Resuscitation, ~4.7 hours from Baseline
FR 170	170 minutes from the start of Full Resuscitation, 3 hours 50 minutes from the start of Limited Resuscitation, ~4.83 hours from Baseline
FR 3	3 hour from the start of Full Resuscitation, 4 hours from the start of Limited Resuscitation, ~5 hours from Baseline
FR 190	190 minutes from the start of Full Resuscitation, 4 hours 10 minutes from the start of Limited Resuscitation, ~5.2 hours from Baseline
FR 4	4 hour from the start of Full Resuscitation, 5 hours from the start of Limited Resuscitation, ~6 hours from Baseline
FR 5	5 hour from the start of Full Resuscitation, 6 hours from the start of Limited Resuscitation, ~7 hours from Baseline
FR 6	6 hour from the start of Full Resuscitation, 7 hours from the start of Limited Resuscitation, ~8 hours from Baseline
FR 7	7 hour from the start of Full Resuscitation, 8 hours from the start of Limited Resuscitation, ~9 hours from Baseline
FR 20	20 hour from the start of Full Resuscitation, 21 hours from the start of Limited Resuscitation, ~22 hours from Baseline